Eur J Cancer Clin Oncol, Vol. 24, No. 11, pp. 1715–1719, 1988.
Printed in Great Britain

0277–5379/88$3.00 + 0.00

Comparison of Enzyme Immunoassay with Dextran-coated Charcoal Method in the Determination of Progesterone Receptor in Breast Cancer Cytosols

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Abstract—*A new enzyme immunoassay (EIA) for progesterone receptor (PR) has been developed by Abbott Laboratories. To study the correlation of the results from this new technique with the currently existing tritiated-ligand binding assay [dextran-coated charcoal (DCC) method], cytosols from 70 human breast cancers were assayed for PR by both the EIA and DCC methods. EIA showed a good reproducibility and was not susceptible to the change in protein concentration of cytosols and types of reducing agents. The correlation between the EIA and DCC methods was excellent with a correlation coefficient of 0.946; regression curve was PR (EIA) = 1.06 × PR(DCC) + 0.19 fmol/mg protein. The concordance of the results obtained from both methods was 91.4% with a cutoff value of 10 fmol/mg protein. These results demonstrate that EIA is a very useful and reliable method in the determination of PR with an excellent correlation with the conventional DCC method.*

INTRODUCTION

IT IS NOW accepted that measurement of the estrogen receptor (ER) and progesterone receptor (PR) in breast cancer is clinically useful for predicting the response of patients to endocrine therapy and for determining their prognosis [1, 2]. These diagnostic assays seem essential for planning the treatment of breast cancer patients.

Several methods have been developed for the determination of ER and PR in breast cancer and all of them are based on the evaluation of the [^{3}H]steroid binding capacity of breast cancer cytosols. These binding assays have been the main methods used during the past 20 years and the clinical significance of ER and PR have been evaluated on the results obtained from these assays. [^{3}H]Ligand binding assays, however, are not devoid of problems. Since these assays can only detect the receptors in the unoccupied form, interference by endogenous hormones cannot be eliminated completely. Another limitation of these assays is that small samples cannot be assayed since a multipoint binding assay usually requires at least 200–300 mg of tissue. Inclusion of tritiated ligands in the assay procedure is another demerit and is not suited for the routine laboratory assay.

Accepted 22 June 1988.
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Recently, an ER enzyme immunoassay (ER-EIA) kit has been introduced and an excellent correlation between the dextran-coated charcoal (DCC) method and ER-EIA has been demonstrated [3, 4]. The ER-EIA kit presently available is based on the direct recognition of the ER molecule by a monoclonal antibody raised against ER purified from MCF-7 cells. The merits of ER-EIA are as follows: (1) ER-EIA can detect ER whether or not it is occupied by endogenous estrogens, this advantage has enabled the application of this method to the exchange assay of ER [5]; (2) only a small sample volume is required for the assay and even specimens obtained from fine needle biopsy or drill biopsy can be assayed accurately with this method [6]; (3) exclusion of tritiated ligands from the assay procedure makes ER-EIA more suited for routine laboratory examination.

More recently, Greene *et al.* have succeeded in producing monoclonal antibody against PR purified from T47D cells [7, 8] and Abbott Laboratories has produced a PR-EIA kit based on this monoclonal antibody, which was demonstrated to recognize both the 95 K and 120 K forms of PR. If PR-EIA can detect PR in cytosols from human breast cancer with accuracy, it will confer the same advantages over conventional DCC method as described for ER-EIA. However, this point has not been studied sufficiently.

In this paper, a comparison of PR-EIA with the conventional DCC method was made on cytosols from 70 human breast cancers. The reliability of

the PR-EIA kit and influence of various assay conditions on PR-EIA results were also studied.

MATERIALS AND METHODS

Preparation of cytosols from surgical specimens

Seventy patients with operable breast cancer were included in this study. Surgically obtained breast cancer specimens were stripped of blood and necrotic tissue and snap frozen in a deep freezer at −80°C. These specimens were kept in this condition until the assay, which was performed in no more than 3 months after the surgery.

All procedures were carried out at 0 – 4°C, unless noted otherwise. The specimens were minced with scissors and homogenized in seven volumes of homogenization buffer [10 mM Tris, 1.5 mM EDTA, 5 mM monothioglycerol, 10 mM sodium molybdate, 10% (v/v) glycerol, pH 7.4] using a Polytron PT homogenizer (Brinkmann Instruments, Inc., Westbury, NY) set at 4, with three 10-s runs allowing 30 s for cooling between each run. The homogenate was centrifuged at 105,000 ***g*** for 60 min and supernatant cytosol was obtained without the superficial lipid layer. Cytosols were diluted with homogenization buffer so that they contained a protein concentration of 1–2 mg/ml.

Tritiated-ligand binding assay for PR (dextran-coated charcoal method)

The cytosol was incubated with 0.1–10 nM (17α-methyl-[^{3}H])-promegestone (86.3 Ci/mmol, New England Nuclear, Boston, MA) in the presence and absence of a 250-fold molar excess of cold promegestone for 18 h (reaction volume, 400 μl). After the incubation, 400 μl of dextran-coated charcoal solution (10 mM Tris, 0.5% Norit A, 0.005% Dextran T-70, pH 8.0) was added and incubated for 30 min with intermittent vortexing. Then, the mixture was centrifuged at 3000 rpm for 10 min and 200 μl of the supernatant was assayed for radioactivity in a liquid scintillation counter. The maximum binding sites and K_d were estimated by Scatchard plot analyses [9].

Enzyme immunoassay for ER and PR

The enzyme immunoassay kit for ER (ER-EIA) was purchased from Abbott Laboratories, Chicago, IL. ER-EIA is a solid phase enzyme immunoassay based on the 'sandwich' principle. All the procedures for the ER-EIA assay were performed according to the manufacturer's instructions. In brief, cytosols, controls and standards were incubated with beads coated with anti-ER monoclonal antibody (rat) for 18 h at 4°C and unbound material was removed by aspiration and washing of the beads with distilled water. A second anti-ER monoclonal antibody (rat) conjugated horseradish peroxidase was incubated with the ER-bead complex at 37°C for 1 h. After aspiration of excess conjugate and washing of the beads with distilled water, the beads were incubated in enzyme substrate (hydrogen peroxide and *o*-phenylenediamine) for 30 min at room temperature. The reaction was stopped by the addition of 1 N sulfuric acid. The intensity of color developed was recorded at 492 nm using a Quantum II. A standard curve was obtained by plotting the absorbance of the standards vs. their ER concentrations, and ER concentrations of samples were determined from this standard curve.

Enzyme immunoassay kit for PR (PR-EIA) was a generous gift from Dainabot Co. Ltd. (Tokyo, Japan). All the assay procedures were performed according to the manufacturer's instructions. The principle on which PR-EIA is based is identical to that of ER-EIA and the assay procedure of PR-EIA is quite similar to that of ER-EIA except for only the difference in temperature (4°C) at the second incubation.

Miscellaneous assays

The protein concentrations were assayed according to the method of Lowry *et al.* [10].

RESULTS

Reproducibility of ER-EIA and PR-EIA

Assay reproducibility was determined by assaying one control (included in each kit) in quintuplicate in the same run (intra-assay variation) and in duplicate in five independent runs (inter-assay variation). The coefficient of variation (% CV) was determined from the components of variance. The excellent reproducibility of ER-EIA has already been reported by many investigators and our results (Table 1) are compatible with those reported by others. % CV of PR-EIA (intra-assay variation, 5.6%; inter-assay variation, 8.7%) were as low as that of ER-EIA (intra-assay variation, 4.1%, inter-assay variation, 7.9%). Therefore, the PR-EIA kit was considered to have an excellent reproducibility identical to that of ER-EIA.

Influence of protein concentration on PR-EIA

The influence of protein concentration of cytosols on the assay results of PR-EIA was studied (Fig. 1). Each cytosol from 26 breast cancers was diluted three-fold with homogenization buffer, and assayed both in the diluted and non-diluted forms by PR-EIA. The mean value of each cytosol (fmol/mg protein) was taken as 100% and the percentage deviation from the mean was plotted in relation to protein concentration (0.5–6.0 mg/ml). A good stability of assay results was observed over this range of protein concentrations.

Table 1. *Intra- and inter-assay variation of ER-EIA and PR-EIA*

	ER-EIA	PR-EIA
Intra-assay variation*		
Mean	118.2	57.4
S.D.	4.8	3.2
% CV	4.1	5.6
Inter-assay variation†		
Mean	122.4	55.2
S.D.	9.6	4.8
%CV	7.9	8.7

*Intra-assay variation was determined by assaying one control in quintuplicate in the same run.
†Inter-assay variation was determined by assaying one control in duplicate in five independent runs.

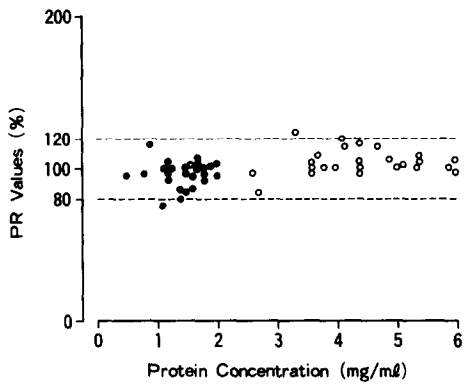

Fig. 1. *Each cytosol from* 26 *breast cancers was diluted with homogenization buffer three-fold and was assayed in the diluted* (●) *and non-diluted* (○) *forms by PR-EIA. The mean result of each cytosol was taken as* 100% *and the percentage deviation from the mean was plotted in relation to protein concentration.*

Influence of reducing agents on PR-EIA

Reducing agents are usually used in the homogenization buffer in conventional tritiated-ligand binding assays in order to stabilize and protect receptor molecules. As reducing agents, monothioglycerol and dithiothreitol are most commonly used. We studied the influence of the reducing agents on PR-EIA (Fig. 2). In this experiment, ten tumors were homogenized in seven volumes of reducing-agent-free buffer [10 mM Tris, 1.5 mM EDTA, 10 mM sodium molybdate, 10% (v/v) glycerol, pH 7.4] and supernatant cytosols were obtained after the centrifugation at 105,000 ***g*** for 60 min. Each cytosol was diluted two-fold with the homogenization buffer containing 10 mM monothioglycerol or 1 mM dithiothreitol and then assayed for PR by PR-EIA. The correlation between PR content obtained from monothioglycerol-containing buffer and those obtained from dithiothreitol-containing buffer is illustrated in Fig. 2. The results obtained under both conditions were almost identical (relative coefficient was 0.991 and regression curve was

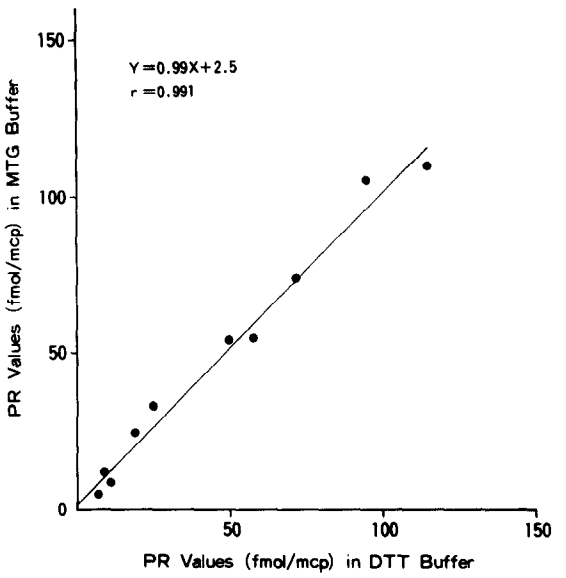

Fig. 2. *Ten cytosols were assayed by PR-EIA in both monothioglycerol* (*MTG*, 5 mM) *containing buffer and dithiothreitol* (*DTT*, 0.5 mM) *containing buffer. Linear regression analysis gave the following results: correlation coefficient* (r) = 0.991 *and regression curve*; y = 0.991x + 2.5. mcp; mg *cytosol protein.*

$y = 0.99x + 2.5$). Therefore, a difference in the reducing agents did not seem to influence PR-EIA.

Influence of receptor occupancy by hormones on EIA

The influence of estrogen binding to ER and progesterone binding to PR on EIA results was studied by incubating five cytosols with 10 nM 17β-estradiol or 10 nM promegestone for 2 h before the ER-EIA or PR-EIA (Table 2). ER contents obtained in the presence and absence of cold 17β-estradiol were almost identical and PR contents obtained in the presence and absence of promegestone were also almost identical. Therefore, PR-EIA, like ER-EIA, is considered to detect PR molecules independent of endogenous progesterone binding.

Correlation between the DCC method and PR-EIA

Cytosols from 70 breast cancers were assayed for PR by both the DCC method and the PR-EIA. The correlation of PR contents obtained from both methods is illustrated in Fig. 3. An excellent correlation was found between PR-EIA and DCC method ($r = 0.946$) and regression curve was $Y = 1.06X + 0.19$ (Y, PR-EIA; X, DCC method).

Regression curves calculated according to the menopausal status were as follows; $Y = 1.13X + 4.53$ ($r = 0.948$) for 31 premenopausals and $Y = 0.99X - 1.13$ ($r = 0.957$) for 39 postmenopausals. No significant difference was found in the slope of regression curves between premenopausals and postmenopausals.

DISCUSSION

Our results demonstrate that the PR-EIA kit has

Table 2. ER and PR values assayed by EIA before and after treatment with estradiol and R5020, respectively

Sample	Treatment with R5020		Treatment with estradiol	
	Before	After	Before	After
1	108*	95	103	90
2	95	92	66	87
3	48	51	26	28
4	11	8	13	12
5	0	0	0	0

*Mean (fmol/mg protein) of triplicate determinations.

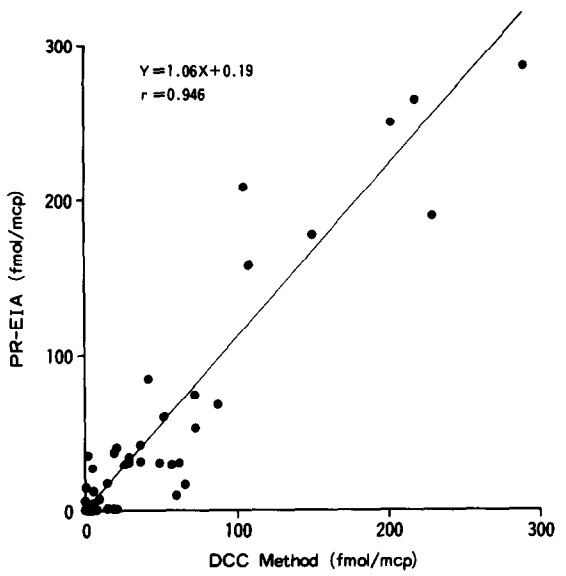

Fig. 3. Cytosols from 70 breast cancers were assayed for PR by both the PR-EIA and DCC methods. Correlation of PR values obtained from both methods is illustrated. Linear regression analysis gave the result of correlation coefficient (r) = 0.946 *and regression curve*; y = 1.06x + 0.19. mcp; mg *cytosol protein.*

good reproducibility and is not susceptible to change in protein concentration of cytosols and types of reducing agents routinely used in the homogenization buffer and that the correlation between tritiated-ligand binding assay and EIA for progesterone receptor in breast cancer cytosols is excellent with a correlation coefficient of 0.946; the slope of the regression curve is 1.06 with the *Y*-axis intercept not significantly different from zero. Therefore, PR-EIA appears to be a very useful method for PR assay with advantages over the conventional DCC method because of the small sample volume required for the assay and the elimination of the need to use radioisotopes in the assay procedure.

Another important advantage of EIA lies in its ability to detect both occupied and unoccupied receptors, in contrast to the DCC method which can measure only unoccupied receptors. With tumors from premenopausal patients, some receptors are thought to be occupied by endogenous hormones, and in such cases the DCC method seems to underestimate the level of receptors while EIA can detect the precise level. Goussard *et al.* reported that ER levels of tumors measured by ER-EIA was significantly higher than those measured by the DCC method in premenopausal patients though ER levels measured by ER-EIA were close to those measured by the DCC method in postmenopausal patients [3]. However, the present results concerning PR assay suggest that endogenous progesterones do not seem to play an important role since no significant difference was found in PR levels measured by both methods irrespective of menopausal status. This result is partly attributable to the difference in exchangeability between ER and PR. Estrogen bound to ER exchanges with [^{3}H]estradiol at 30°C, but not at 0–4°C where the DCC method for ER assay is usually performed [11]. Therefore, when there are some occupied ERs, the DCC method may underestimate the ER value since it can only detect unoccupied ERs. On the other hand, PR is known to release progesterone more easily than ER does estrogen even at 0–4°C. Bayard *et al.* demonstrated that progesterone bound to PR easily exchanges with [^{3}H]progesterone at 0–4°C [12]. Even in the presence of occupied PRs, the DCC method is considered to detect both occupied and unoccupied PRs. Therefore, interference of endogenous progesterones are less likely to occur in the PR assay as compared to the ER assay.

ER-EIA has been demonstrated to be well suited for the determination of nuclear ER (ERn) in high salt extracts of myofibrillar pellets [5]. Since conventional exchange assays for ERn are sensitive to procedural details and have unavoidable problems such as ER degradation during assay and relatively large amounts of tissue required for the assay, ER-EIA, which is almost devoid of the above-mentioned problems, will be a very useful tool for an accurate determination of ERn. Probably, PR-EIA can also be used for a PRn assay because PR-EIA can detect both unoccupied and occupied forms of PR and high salt conditions, which are necessary for the extraction of PRn from myofibrillar pellets, do not affect PR-EIA (data not shown).

DCC methods currently in use in laboratories vary from one to another in procedural details such as composition of buffer and incubation conditions. This situation is inconvenient for the interlaboratory comparison of PR data. However, PR-EIA will solve this problem due to a total standardization of assay procedures. Moreover, a correlation line between the PR-EIA and DCC methods obtained in our series was approximately 1.0 with a *Y*-axis intercept not significantly different from zero. Therefore, serious clinical problems are unlikely to occur in the interpretation of PR data after the passage of the DCC method to PR-EIA. Due to the excellent reproducibility and the other advantages mentioned above, PR-EIA seems likely to replace the DCC method in the future.

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